Identification of Rat Targets of Anti-Soluble Liver Antigen Autoantibodies by Serologic Proteome Analysis

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Background: Anti-soluble liver antigen (SLA) autoantibodies are specific for autoimmune hepatitis type 1 and are the only immunologic marker found in 15–20% of hepatitis cases previously considered cryptogenic. Anti-SLA antibodies react with the 100 000 g supernatant from rat liver homogenate, but the molecular targets remain controversial.

Methods: We characterized anti-SLA targets by one- and two-dimensional immunoblotting analysis. The recognized proteins were identified by peptide mass fingerprint analysis after matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Results: Three proteins of 35 kDa and pI 6.0, 50 kDa and pI between 6.0 and 6.5, and 58 kDa and pI between 6.5 and 7.0 were stained more intensely by anti-SLA positive-sera than by control sera. After in-gel tryptic digestion, MALDI-TOF analysis of the generated peptides enabled the clear identification of N-hydroxyarylamine sulfotransferase, isoforms of α-enolase, and isoforms of catalase.

Conclusions: Possible antigens for anti-SLA antibodies include a sulfotransferase, α-enolase(s), and catalase(s). Two-dimensional electrophoresis combined with mass spectrometry offers a versatile tool to identify molecular targets of autoantibodies and thus to improve diagnostic tools and the understanding of the immune process.

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Autoimmune hepatitis (AIH),4 a liver disease of unknown etiology, accounts for ~20% of chronic hepatitis cases in the Caucasian population and responds well to immunosuppressive therapy (1). The diagnosis of AIH remains difficult and relies on the exclusion of other causes of chronic hepatitis and use of an international scoring system that includes epidemiologic, biochemical, histologic, and serologic criteria (2).

Autoantibody profiles distinguish subtypes of AIH (2,3). AIH type 1 (AIH-1), the most common form, is characterized by the presence of anti-nuclear antibodies (ANAs) and/or anti-smooth muscle antibodies (SMAs) with actin specificity, whereas AIH type 2 (AIH-2) is associated with antibodies to liver-kidney-microsome type 1 (LKM1) and/or antibodies to liver cytosol type 1 (LC1). However, these four antibodies are not specific to AIH as they are also found in sera from patients with viral hepatitis, drug-induced hepatitis, or other autoimmune diseases (4–7). In contrast, antibodies to soluble liver antigen (SLA) have been shown to be an uncommon but very specific marker of AIH-1 (8–10), allowing the reclassification of 15–20% of hepatitis cases negative for other antibodies, i.e., cryptogenic hepatitis, with substantial prognostic and therapeutic implications (9,11).

To better understand the pathogenesis of AIH and to establish a simpler and more reliable diagnostic test than the complex reference ELISA, many controversial studies have described the target antigens of anti-SLA antibodies. Cytokeratins 8/18 (52/45 kDa) were initially suggested as targets (12), but it is now well established that these

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5 Received August 6, 2002; accepted January 23, 2003.

4 Nonstandard abbreviations: AIH-1 and -2, autoimmune hepatitis type 1 and 2; ANA, antinuclear antibody; SMA, smooth muscle antibody; LKM1, liver-kidney-microsome type 1; LC1, liver cytosol type 1; SLA, soluble liver antigen; GST, glutathione S-transferase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; BSA, bovine serum albumin; IIF, indirect immunofluorescence; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEF, isoelectric focusing; IPG, immobilized pH gradient; DTT, dithiothreitol; and HAST-1, N-hydroxyarylamine sulfotransferase type 1.
cytokeratins are not components of SLA (13, 14). The reactivity of anti-SLA-positive sera with 25- to 27-kDa subunits of glutathione S-transferase (GST) has also been reported (15, 16), especially against the Ya subunit (17). Recently, two immunoreactive cDNA clones encoding for a 50-kDa gene product and its N-terminal-truncated form of 35 kDa were proposed as anti-SLA targets (14, 18). The 50-kDa recombinant protein was identified as a UGA-serine tRNA-associated protein [tRNA[Ser]Sec] (14, 19). However, this recombinant antigen raises some questions, because only approximately three-fourths of anti-SLA-positive sera produced a positive reaction when tested with this tRNA[Ser]Sec-associated protein (18). Furthermore, previous studies had indicated that SLA constitutes a mixture of distinct antigenic proteins (12, 15).

The aim of this study was to use a serologic proteomic analysis, including one- and two-dimensional gel electrophoresis, immunoblot analysis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the reliable characterization and identification of putative targets of anti-SLA antibodies.

Materials and Methods
All chemical reagents used were of research grade and obtained from Sigma-Aldrich, unless otherwise stated.

Antigen Preparation
Livers from Wistar male rats (Janvier breeding facility, le Genet Saint-Isle, France) were homogenized with a Potter-Elvehjem apparatus in 10 mmol/L Tris-HCl (pH 7.4), 250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L 4-(2-aminooethyl)benzenesulfonyl fluoride (AEBSF). Cellular fractions were prepared by differential centrifugation as described elsewhere (20), aliquoted, and stored at −80 °C until use. Protein concentrations were measured using the bicinchoninic acid method (Pierce) with bovine serum albumin (BSA) as calibrator.

Autoantibody Testing
Sera were screened for ANAs and anti-LKM1, anti-LC1, and anti-SMA autoantibodies by indirect immunofluorescence (IIF) on rat liver, kidney and stomach cryostat sections, as described elsewhere (21, 22). Anti-actin cable antibodies were detected in sera with anti-SMA autoantibodies by IIF on colchicine-treated Hep2 cell monolayers (21). Anti-LC1 and anti-LKM1 autoantibodies were confirmed by the Ouchterlony method, as described previously (22).

Not detectable by IIF (23), anti-SLA antibodies were identified by inhibition ELISA, according to the method described by Grüber et al. (24). The anti-SLA-positive reference serum, a gift from Prof. Dr. Manns (Abteilung Gastroenterologie und Hepatology, Medizinische Hochschule, Hanover, Germany), was first saturated with ammonium sulfate to obtain the immunoglobulin fraction. A first aliquot (20 mg/L) was labeled with biotinamidocaproate N-hydroxysuccinimide ester and solubilized in dimethyl sulfoxide. A second aliquot, adjusted to 20 mg/L in phosphate-buffered saline (PBS), was used to coat microtiter plates (Dynatech Laboratories) at 50 μL/well.

After the plates were washed, 50 μL of the 100 000g supernatant of rat liver homogenate (100 mg/L) was added to each well. After incubation for 1 h and extensive washing, the plates were incubated for 1 h with 50 μL of the test sera, anti-SLA-positive serum, and negative controls, each in duplicate at a dilution of 1:10 (5 μL of serum in 45 μL of PBS-EDTA), as used by other authors (11, 14, 24).

After being washed, the wells were incubated for 45 min with 50 μL of the biotin-labeled immunoglobulins from the anti-SLA-positive reference serum, diluted 1:1500 (10 μL of labeled immunoglobulins in 15 mL of PBS containing 10 g/L BSA). The binding of these labeled anti-SLA antibodies was detected with avidin-peroxidase (50 μL/well; Dako), diluted 1:100 (100 μL of avidin-peroxidase in 10 mL of PBS-BSA), with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. The results were expressed as the inhibition (as a percentage) of the binding of labeled anti-SLA antibodies to their specific antigens. A serum was considered positive if it inhibited the binding of biotinylated anti-SLA globulins by at least 40%. The 40% cutoff is well above the mean + 2 SD of inhibition obtained with sera from healthy blood donors; the inhibition cutoff was the same as that used by other authors (8, 14, 18, 24).

Participants and Blood Samples
We selected 44 anti-SLA-positive sera for one-dimensional immunoblotting analysis: 19 were positive only for anti-SLA antibodies, 15 were positive for both anti-SLA and anti-actin cable antibodies, 6 were positive for both anti-SLA antibodies and ANAs, and 4 were positive for anti-SLA and anti-actin cable antibodies and ANAs. In the anti-SLA-negative control group (n = 70), we investigated 20 sera from AIH-1 patients, 5 from AIH-2 patients, and 45 sera from healthy individuals. All patients fulfilled the criteria for AIH, as defined by the International Autoimmune Hepatitis Group (2). For two-dimensional immunoblotting analysis, we selected six anti-SLA-positive sera that gave typical one-dimensional reactivity patterns and six anti-SLA-negative controls (two from AIH-1 patients and four from healthy individuals). Five of the six anti-SLA-positive sera were negative for ANAs and anti-SMA antibodies, and one was positive for both antibodies.

All sera were collected between January 1997 and January 2001 in the Immunological Laboratory of Saint-Antoine Hospital and stored at −80 °C until being assayed.

One-dimensional Gel Electrophoresis
The 100 000g supernatant of liver homogenate was boiled for 3 min in sample buffer [62.5 mmol/L Tris-HCl, pH 6.8, containing 20 g/L sodium dodecyl sulfate (SDS), 1 mL/L 2-mercaptoethanol, 90 mL/L glycerol, and a trace of...
pyronine]. One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 200 μg of protein per centimeter was performed according to the method described by Laemmli (25) in a Protein II slab gel vertical electrophoresis unit (Bio-Rad) with a 4% polyacrylamide staking gel and a 10% separating gel. Protein molecular weights were assigned according to the low-range molecular weight calibrator from Bio-Rad.

**TWO-DIMENSIONAL GEL ELECTROPHORESIS**

Isoelectric focusing (IEF) was performed with a ready-made immobilized pH gradient (IPG) system (26), i.e., Immobiline DryStrips (110 mm, pH 4–7 or pH 6–11; Amersham Pharmacia Biotech). We first solubilized 600 μg of the 100 000g supernatant in a solution containing 20 g/L CHAPS, 3 g/L dithiothreitol (DTT), and 5 mmol/L AEBSF. After boiling for 3 min, proteins were precipitated by ice-cold acetone for 30 min.

After centrifugation (17 000g for 15 min), the pellet was suspended in 10 mol/L urea containing 20 g/L CHAPS, 3 g/L DTT, 5 mL/L IPG buffer, pH 4–7 or pH 6–11 (Amersham Pharmacia Biotech), and a trace of Orange G. Samples were applied by cup-loading, and IEF was performed at 20 °C in a horizontal electrophoresis apparatus (Multiphor II; LKB). Focusing was started at 150 V, and the voltage was gradually increased to 3 kV to reach a total of 40 kV·h for IPG, pH 4–7, and 60 kV·h for IPG, pH 6–11. After IEF, strips were equilibrated twice for 15 min each in 50 mmol/L Tris-HCl, pH 6.8, containing 6 mol/L urea, 300 mL/L glycerol, and 20 g/L SDS, supplemented first with 5 g/L DTT for protein reduction and then with 45 g/L iodoacetamide for carbamidomethylation, plus a trace of bromphenol blue. Equilibrated IPG strips were loaded on the upper edge of the vertical SDS gel, and the second-dimension electrophoresis was carried out at constant current of 20 mA per gel for stacking and 40 mA for the resolving gel. After separation, the proteins were either stained with colloidal Coomassie brilliant blue (to obtain a protein map) or transferred to a nitrocellulose membrane. The two-dimensional stained gels were analyzed with Image Master 3.0.1 software (Amersham Pharmacia Biotech). Gels used for immunoblotting were silver-stained after transfer (PlusOne silver staining reagent; Amersham Pharmacia Biotech).

**IMMUNOBLOTTING**

Proteins separated by one- and two-dimensional electrophoresis were transferred to nitrocellulose membranes (Bio-Rad) for 2 h at 60 V, according to the method described by Towbin et al. (27), in a Trans-Blot cell (Bio-Rad). The membranes were then blocked with 50 g/L nonfat milk in 150 mmol/L PBS and probed for 3 h at 20 °C with sera diluted 1:100 (30 μL of serum in 3 mL of PBS containing 1 mL/L Tween® 20). After extensive washing and a 1.5-h incubation with peroxidase-conjugated goat anti-human immunoglobulin (Bio-Rad), bound antibodies were detected by adding as a substrate 0.6 g/L 4-chloro-1-naphthol (Merck) diluted in 200 mL/L methanol containing 0.033 mL/L H2O2.

To elute anti-SLA antibodies from their targets on one-dimensional immunoblots, the stained bands were cut out from the nitrocellulose sheets and dipped in a 0.2 mol/L glycine buffer, pH 2.2, containing 0.5 mol/L NaCl and 10 mL/L Tween 20. The pH of the eluate was quickly neutralized with 1 mol/L Tris. The eluted antibodies were then dialyzed against PBS containing 10 mol/L EDTA.

Colloidal Coomassie blue-stained protein maps, silver-stained transferred two-dimensional gels, and nitrocellulose membranes were scanned and superimposed with use of Adobe Photoshop to localize recognized protein spots.

**ADSORPTION STUDIES**

We mixed 0.5-mL aliquots of two anti-SLA-positive sera with characteristic staining patterns on immunoblotting with 20 mg of nuclei, mitochondria, microsomes, or the 100 000g supernatant fractions from rat liver. After incubation for 18 h at 4 °C, the mixtures were centrifuged at 100 000g for 30 min. The process was repeated twice with the supernatant obtained after each centrifugation. The last supernatant was tested by immunoblotting with the 100 000g fraction as the antigen.

**PROTEIN AND PEPTIDE PREPARATION PROCEDURES**

Proteins were prepared for MS under a laminar flow exhaust hood. Colloidal blue-stained antigenic spots were excised from two-dimensional gels and cut into ~1 mm² pieces. The protein spots were digested in the gel slices with trypsin (sequencing grade, EC 3.4.21.4; Roche Diagnostics GmbH) as described by Shevchenko et al. (28). Spots of interest were washed successively in distilled water, acetonitrile, and 100 mmol/L NH4HCO3 before being immersed under reduced pressure in a Speedvac centrifuge (Savant Instruments). After the reduction of cysteine (10 mmol/L DTT–100 mmol/L NH4HCO3 for 45 min at 56 °C) and protein alkylation (55 mmol/L iodoacetamide–100 mmol/L NH4HCO3 for 30 min in the dark at room temperature), the gel pieces were washed successively with 100 mmol/L NH4HCO3, a 1:1 (by volume) mixture of 100 mmol/L NH4HCO3 and acetonitrile, and acetonitrile and dried again. The gel pieces were then rehydrated for 45 min at 4 °C in a digestion buffer containing 50 mmol/L NH4HCO3, 5 mol/L CaCl2, and 12.5 mg/L trypsin. Excess protease was removed, and the volume was adjusted with 25 mmol/L NH4HCO3 to cover the gel pieces. Digestion was allowed to proceed overnight at 37 °C.

After trypsin digestion, the resulting peptide mixtures were extracted by incubation in 10 g/L formic acid for 15 min, followed successively by two extractions with 10 g/L formic acid–acetonitrile (1:1 by volume) and acetonitrile. Peptide extracts were pooled and dried in the vacuum centrifuge. The peptides were then solubilized with 10 μL of formic acid and desalted with ZipTip® C18 pipette tips (Millipore). The tips were washed with 10 g/L formic
acid, and the peptides were eluted with 4 μL of a 1:1 (by volume) mixture of 10 g/L formic acid–acetonitrile and 4 μL of a 2:8 mixture (by volume) of 10 g/L formic acid–acetonitrile. The desalted peptides were dried under reduced pressure in a Speedvac centrifuge.

**Peptide Mass Fingerprint Using MALDI-TOF MS**

After each peptide sample was dissolved in 3 μL of 10 g/L formic acid, 0.5 μL was applied to the sample plate and mixed with the same volume of the supernatant of a saturated 2,5-dihydroxybenzoic acid solution (dried droplet method). MALDI-TOF spectra (600–4000 Da) were acquired in the positive reflectron mode with a Voyager DE-STR Biospectrometry Workstation mass spectrometer (Applied Biosystems) with an accelerating voltage of 20 kV (delayed extraction time, 200 ns; ~250 scans averaged for each acquisition). The spectra were calibrated externally using the [M+H]⁺ ion from Des-Arg-bradykinin (904.46 Da) and adrenocorticotrophin peptide (clip 18–39; 2465.20 Da). Trypsin autoproteolysis products (658.39, 805.42, 1153.57, 2163.06, and 2273.16 Da) were used as the second internal standard. For subsequent data processing, Data Explorer software (Applied Biosystems) was used. Data mining was performed using the ProFound search program (29). The ProFound search program (30) was used with following parameters: mammalian species, molecular mass range from 10 to 100 kDa, pI range from 0 to 14, monoisotopic peptide masses, and two missed cleavages by trypsin, and a mass deviation of 50 ppm was allowed in nonredundant SWISS PROT database searches. Partial chemical modifications, such as the oxidation of methionine and the carbamidomethylation of cysteine, were taken into consideration for any queries.

**Statistical Analysis**

Qualitative data were compared using the χ² test, with the Yates correction if necessary. The minimum level of significance was set at P < 0.05.

**Results**

**One-Dimensional Reactivity Patterns of Anti-SLA Antibodies**

Examples of the one-dimensional reactivity patterns for anti-SLA-positive and negative-control sera against pro-

**Fig. 1.** Electrophoretic pattern of 100 000g supernatant proteins from rat liver homogenate (left) and representative immunoblotting patterns of anti-SLA-positive and -negative sera (right).

(Left), the 100 000g supernatant proteins from rat liver homogenate (right lane) and molecular mass markers (left lane) were separated by SDS-PAGE and stained with colloidal Coomassie brilliant blue (CB). (Right), lanes 1–7 show representative immunoblotting patterns of anti-SLA-positive sera; lanes 8–13 show representative patterns for the anti-SLA-negative group: lanes 8–10, blood donors; lanes 11 and 12, anti-SLA-negative AIH-1; lane 13, anti-LC1-positive AIH-2.

**Fig. 2.** Frequencies of common bands stained by controls and anti-SLA-positive sera in immunoblotting analysis performed with rat liver 100 000g supernatant as the antigen. Sera were diluted 1:100. Three protein bands of 58, 50, and 35 kDa were stained by anti-SLA-positive sera with a statistically significant frequency compared with the anti-SLA-negative group. P values indicate the results of the χ² test. MW, molecular weight; NS, not significant.
Proteins separated from rat liver 100 000g supernatant are shown in Fig. 1. Anti-SLA-positive sera reacted significantly with at least one protein band at 50, 58, or 35 kDa. More precisely, 30 of 44 (68%) anti-SLA-positive sera stained a 50-kDa protein vs 6 of 70 (8.5%) control sera ($P < 0.0001$), 14 of 44 (29%) anti-SLA-positive sera stained a 35-kDa protein vs 5 of 70 (7%) controls ($P < 0.001$), and 14 of 44 (29%) anti-SLA-positive sera stained a 58-kDa protein vs 3 of 70 (4%) controls ($P < 0.001$; Fig. 2). Some anti-SLA-positive sera weakly stained other protein bands (Fig. 1), especially at 25–27 kDa (36%) and 42 kDa (9%), but the amount of staining did not differ significantly from that for the control group (21% and 6%, respectively; Fig. 2). Eight of 44 (18%) anti-SLA-positive sera had no reaction on one-dimensional immunoblots (Fig. 2).

Antibodies from two sera staining the three bands, i.e., 58, 50, and 35 kDa, were eluted from the nitrocellulose sheets. The protein concentrations were adjusted to be the same as the immunoglobulin concentrations in the sera. The inhibition of eluted antibodies, measured by inhibition ELISA, was >40%, indicating a positive reaction.

No bands at 58, 50, and 35 kDa were stained by anti-SLA-positive sera on immunoblots performed with nuclear, mitochondrial, or microsomal fractions (Fig. 3).

Adsorption of anti-SLA-positive sera with the 100 000g supernatant dramatically decreased the reactivity on immunoblotting with this fraction, whereas adsorption with the other fractions had no effect (Fig. 3).

**TWO-DIMENSIONAL IMMUNOBLOTTING ANALYSIS OF ANTI-SLA-POSITIVE SERA**

The Coomassie-stained two-dimensional patterns of the rat liver cytosolic fraction are shown in Fig. 4. The pH 4–7 gradient (Fig. 4A) resolved 264 protein spots homogeneously distributed throughout the gel, whereas the pH 6–11 gradient (Fig. 4B) showed 165 spots located in the more acidic region. The method was highly reproducible with several gels. Representative examples of the two-dimensional immunoblotting reactivity of anti-SLA antibodies against proteins from the 100 000g supernatant are shown in Fig. 5.
The three significantly stained bands of 50, 35, and 58 kDa on one-dimensional immunoblots corresponded on two-dimensional immunoblots to four contiguous protein spots located between experimental pI 6.0 and 6.5 (Fig. 5B), one spot at pI 6.2 (Fig. 5B), and three spots between pI 6.5 and 7.0 (Fig. 5A), respectively. The protein spots stained by controls were systematically different from those targeted by anti-SLA-positive sera (data not shown).

**PROTEIN IDENTIFICATION**

Targeted protein spots from two experiments were analyzed by MALDI-TOF MS. Representative examples of the mass spectra generated by analysis of each group of target antigens are shown in Fig. 6. As for identification by peptide mass fingerprinting, three variables were considered: the number of peptides in the peptide fingerprint matching one given protein in the database; the probabil-
ity of the match calculated by the ProFound program; and sequence coverage by the matched peptides. It was considered that the latter needed to be >20% for correct identification by MALDI-TOF MS.

The data for proteins identified by MALDI-TOF MS in one experiment are listed in Table 1; the data resulting from a second experiment were identical. Table 1 shows the spot number corresponding to either Fig. 5A or 5B, the protein name, and the corresponding accession code. In addition, the theoretical and observed masses and pI values are given. The data listed in the “Peptides matched” column in Table 1 are based on MS analyses, and the last column indicates the percentage sequence coverage. ProFound results furnished unambiguous identifications with a 100% probability for each spot except spot 2 (keratin contamination). Antigens of 50, 35, and

Fig. 6. Representative peptide mass fingerprints obtained by MALDI-TOF MS of the tryptic digests of spots corresponding to immunoreactive peptides of 58 kDa (A), 50 kDa (B), and 35 kDa (C). *
* indicate matching peptides.
58 kDa, respectively, were identified as isoforms of α-enolase, N-hydroxyarylamine sulfotransferase type 1 (HAST-1), and isoforms of catalase, all from the Rattus norvegicus species. Differences between the theoretical peptide mass and the observed peptide mass were all ≤50 ppm. Most sequence coverages were >54%. The molecular masses and the pI values of the identified proteins agreed well with the theoretical masses and pI values of the matched proteins.

**Discussion**

The present study confirms that anti-SLA antibodies are heterogeneous, as suggested more than a decade ago by Wächter et al. (12). The large number of sera tested by one-dimensional immunoblots (n = 44) compared with the results obtained by Wächter et al. (n = 15) (12) enabled us to highlight three bands stained at a statistically significant frequency compared with controls, i.e., 58 kDa (29% vs 4%), 50 kDa (68% vs 8.5%), and 35 kDa (29% vs 7%). Wesierska-Gadek et al. (15) had also reported that SLA might be a mixture of various antigens, but they did not specify their molecular masses.

The identity between anti-SLA antibodies detected on immunoblots and by ELISA was shown by the positive inhibition in the ELISA obtained with the antibodies eluted from the stained bands on nitrocellulose. Nevertheless, 18% of anti-SLA-positive sera were negative on an immunoblot. This observation indicates the existence of conformational epitopes for SLA that were not studied by the immunoblotting method used in the present study.

Not detected on immunoblots performed with nuclear, mitochondrial, or microsomal fractions, the 58-, 50-, and 35-kDa stained bands were present only in the 100 000g supernatant fraction. These reactivities were lost when anti-SLA-positive sera were pre-adsorbed with this 100 000g fraction, whereas pre-adsorption with the cellular organelles had no effect, indicating that the 58-, 50-, and 35-kDa species are restricted to the 100 000g supernatant.

Our identification of immunoreactive spots by proteomic technology led to the identification of potential antigens for anti-SLA antibodies, i.e., four α-enolase isoforms for the 50-kDa antigen, three catalase isoforms for the 58-kDa antigen, and HAST-1 for the 35-kDa antigen.

The glycolytic enzyme α-enolase was significantly recognized by 68% of anti-SLA-positive sera. Antibodies against this antigen have previously been described in AIH (31) but also in a wide spectrum of autoimmune diseases (32), including primary biliary cirrhosis and systemic lupus erythematosus (33). These data suggest an apparent contradiction concerning the high specificity of anti-SLA antibodies for AIH-1. More than 10 different isoforms of α-enolase have been described in yeast, and such heterogeneity has been confirmed in humans (34, 35). In this report, we describe four distinct isoforms of α-enolase from the rat liver cytosol that constitute possible targets of anti-SLA antibodies. In line with other investigators (33, 36), we suggest that antibodies against α-enolase constitute a heterogeneous family sharing different degrees of reactivity toward different isoforms and/or epitopes of α-enolase, depending on the pathologic setting.

As described for the multiple forms of α1-antitrypsin in human plasma (37), the differences in the pI values and molecular masses for the four targeted two-dimensional separated isoforms of α-enolase appeared to be consistent with typical posttranslational modifications attributable to glycosylation. Although it has been shown that glycosyl chemical groups constitute very efficient antigenic epitopes, we suggest that anti-SLA antibodies specifically recognize glycosylated isoforms of α-enolase or exhibit higher affinity for them compared with other isoforms. To validate this hypothesis, we propose that, after the purification of targeted isoforms by two-dimensional preparative electrophoresis, mass spectrometry should be used to characterize the posttranslational modifications of targeted α-enolase and that specific affinity measurements should be performed.

Three distinct catalase isoforms reacted with 29% of anti-SLA-positive sera. Catalase is a protective cellular enzyme that catalyzes the transformation of toxic hydrogen peroxide in water. In the same way as for enolase, this protein has been shown to be an antigenic target for antibodies directed to the neutrophil polynuclear cyto-
plasm in patients with autoimmune liver disease or inflammatory bowel disease, in whom α-enolase antibodies are frequently detected (38, 39). Interestingly, anti-enolase and anti-catalase antibodies, as anti-SLA antibodies, have been described as being undetectable by IIF (23, 39). Affinity measurements coupled with isoform and epitope mapping of both anti-catalase and anti-α-enolase in different pathologies seem to be necessary to obtain a clear understanding of their broad distribution.

HAST-1 was recognized by 29% of anti-SLA-positive sera. To our knowledge, this constitutes the first report describing an immunogenic role for HAST-1. HAST-1 is a hepatic conjugation enzyme implicated in the sulfation of diverse metabolically oxidized N-aryl compounds, including biogenic amines, steroids, hormones, bile acids, and many xenobiotics in the rat (40, 41). Several forms of aryl sulfttransferase have been purified in humans (42).

In liver diseases, autoantibodies directed against enzymes involved in drug detoxification have been described, such as anti-cytochrome antibodies, e.g., anti-CYP2D6 in AIH-2 (2), anti-CYP2C9 in tienilic acid-induced hepatitis (1), and anti-CYP1A2 in dihydralazine-induced hepatitis (1). There have been reports that the complexing of reactive metabolites with the enzyme that forms them may correspond to a modified self-antigen that triggers the autoimmune process (43). A similar mechanism can be evoked for the generation of anti-HAST-1: it has been shown that O-sulfate groups resulting from N-hydroxyarylamine can bind covalently to biological macromolecules (41).

The proteins stained in the molecular mass range of 25–27 kDa with a frequency of 36% were perhaps subunits of the GST described by Wesierska-Gadek et al. (15). The frequency of anti-GST antibodies in anti-SLA-positive sera reported by different authors is 14–80% (15, 16). We did not study anti-GST reactivity by two-dimensional electrophoresis and MS because the difference in frequencies for the 25- to 27-kDa stained bands was not significant between anti-SLA-positive sera and controls.

The authors of two recent independent studies involving immunoscreening of a human cDNA bank reported the protein sequences of two putative anti-SLA antibody targets. The first sequence, with a theoretical molecular mass of 50 kDa and a pI of 9.4, corresponds to a protein associated with a UGA-serine tRNA (14, 19). The second target antigenic sequence, with a theoretical molecular mass of 35 kDa, corresponds to an experimentally N-terminal-truncated form of the previous protein (18). These data contradict the results of previous studies in which two-dimensional immunoblotting of anti-SLA antibodies with human proteins indicated that the 50-kDa targeted protein focused between pI 6.0 and pI 7.5 (12, 13). Our results obtained with rat proteins agree with these findings and suggest that the origin of the preparation may not explain this difference in pI.

Our data may also illustrate a discrepancy between the proteomic and genomic approaches. Using proteomic technology, we investigated antigens at their level of expression in the cell, whereas the immunoscreening of a cDNA bank can predict the amino acid sequence of a protein but does not provide information on either the degree of its expression or its maturation (44). The poor correlation between mRNA abundance and corresponding protein expression is now well established (45). On the other hand, it seems that the number of spots usually separated by two-dimensional electrophoresis is not representative of the overall gene expression (46). Consequently, the tRNA(Ser)Sec-associated protein can exhibit variable or weak expression or posttranslational modifications in hepatocytes, which would mean that it could not be detected during immunoblot experiments with the liver homogenate 100 000g supernatant.

To evaluate the expression of identified antigens, we determined the respective relative intensities of the staining of targeted protein spots, using two-dimensional analysis Image master software. The four isoforms of α-enolase and HAST-1 corresponded to ~3–4% and 1%, respectively, of the total intensity in the pH range 4–7, whereas the three catalase isoforms corresponded to ~3% in the pH range 6–11. These data show that the putative antigens identified were strongly expressed in the rat liver cytosolic fraction and at concentrations well above the detection limit of the 4-chloro-1-naphthol (500 pg) used for antibody detection.

In conclusion, we propose that, based on the combined use of two-dimensional immunoblotting and peptide mass fingerprinting analysis after MALDI-TOF MS, N-hydroxyarylamine sulfotransferase, specific isoforms of α-enolase, and isoforms of catalase are possible antigens for anti-SLA antibodies. Biological tests using recombinant proteins are needed, however, to confirm the reactivity of the proposed anti-SLA antibody targets. MS may also help in a future study to define the posttranslational modifications of these targeted isoforms.

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